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(54) Title: NOVEL ANTIMICROBIAL PEPTIDE, COMPOSITIONS CONTAINING SAME AND USES THEREOF

(57) Abstract

This invention provides a purified polypeptide useful as an antimicrobial agent. This purified polypeptide has been derived from human granulocytes, and has a molecular weight of about 3,700 daltons and the N-terminal amino acid sequence val-cys-ser-cys-arg-leu-val-phe-cys-arg-arg-thr-glu-leu-arg-val-gly-asn-cys-leu-ilu-gly-gly-val-ser-phe-thr-tyr-cys-cys-thr-arg-val. This invention also provides methods for producing this polypeptide, pharmaceutical compositions containing the polypeptide, and uses thereof.

COMPARISON OF PEAK 2 TO HUMAN,
RABBIT, AND GUINEA PIG DEFENSINS

		1				5					10			
PEAK 2		VAL	CYS	SER	CYS	ARG	LEU	VAL	PHE	CYS	ARG	ARG	THR	GLU
HNP-1**		ALA	CYS	TYR	CYS	ARG	ILE	PRO	ALA	CYS	ILE	ALA	GLY	GLU
HNP-2			CYS	TYR	CYS	ARG	ILE	PRO	ALA	CYS	ILE	ALA	GLY	GLU
HNP-3		ASP	CYS	TYR	CYS	ARG	ILE	PRO	ALA	CYS	ILE	ALA	GLY	GLU
RNP-1***	VAL	VAL	CYS	ALA	CYS	ARG	ARG	ALA	LEU	CYS	LEU	PRO	ARG	GLU
RNP-2	VAL	VAL	CYS	ALA	CYS	ARG	ARG	ALA	LEU	CYS	LEU	PRO	LEU	GLU
RNP-3a	GLY	ILE	CYS	ALA	CYS	ARG	ARG	ARG	PHE	CYS	PRO	ASN	SER	GLU
RNP-3b	GLY	ARG	CYS	VAL	CYS	ARG	LYS	GLN	2LEU*	CYS	SER	TYR	ARG	GLU
RNP-4	VAL	SER	CYS	THR	CYS	ARG	ARG	PHE	SER	CYS	GLY	PHE	GLY	GLU
RNP-5	VAL	PHE	CYS	THR	CYS	ARG	GLY	PHE	LEU	CYS	GLY	SER	GLY	GLU
GPNP****	ARG	ARG	CYS	ILE	CYS	THR	THR	ARG	THR	CYS	ARG	PHE	PRO	TYR

		15					20					25		
PEAK 2	LEU	ARG	VAL	GLY	ASN	CYS	LEU	ILE	GLY	GLY	VAL	SER	PHE	THR
HNP-1	ARG	ARG	TYR	GLY	THR	CYS	ILE	TYR	GLN	GLY	ARG	LEU	TRP	ALA
HNP-2	ARG	ARG	TYR	GLY	THR	CYS	ILE	TYR	GLN	GLY	ARG	LEU	TRP	ALA
HNP-3	ARG	ARG	TYR	GLY	THR	CYS	ILE	TYR	GLN	GLY	ARG	LEU	TRP	ALA
RNP-1	ARG	ARG	ALA	GLY	PHE	CYS	ARG	ILE	ARG	GLY	ARG	ILE	HIS	PRO
RNP-2	ARG	ARG	ALA	GLY	PHE	CYS	ARG	ILE	ARG	GLY	ARG	ILE	HIS	PRO
RNP-3a	ARG	PHE	SER	GLY	TYR	CYS	ARG	VAL	ASN	GLY	ALA	ARG	TYR	VAL
RNP-3b	ARG	ARG	ILE	GLY	ASP	CYS	LYS	ILE	ARG	GLY	VAL	ARG	PHE	PRO
RNP-4	ARG	ALA	SER	GLY	SER	CYS	THR	VAL	ASN	GLY	VAL	ARG	HIS	THR
RNP-5	ARG	ALA	SER	GLY	SER	CYS	THR	ILE	ASN	GLY	VAL	ARG	HIS	THR
GPNP	ARG	ARG	LEU	GLY	THR	CYS	ILE	PHE	GLN	ASN	ARG	VAL	TYR	THR

			30			
PEAK 2	TYR	CYS	CYS	THR	ARG	VAL
HNP-1	PHE	CYS	CYS			
HNP-2	PHE	CYS	CYS			
HNP-3	PHE	CYS	CYS			
RNP-1	LEU	CYS	CYS	ARG	ARG	
RNP-2	LEU	CYS	CYS	ARG	ARG	
RNP-3a	ARG	CYS	CYS	SER	ARG	ARG
RNP-3b	PHE	CYS	CYS	PRO	ARG	
RNP-4	LEU	CYS	CYS	ARG	ARG	
RNP-5	LEU	CYS	CYS	ARG		
GPNP	PHE	CYS	CYS			

* leu - leu bond expressed as 2leu
** HNP - human neutrophil peptide
*** RNP - rabbit neutrophil peptide
**** GPNP - guinea pig neutrophil peptide

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NOVEL ANTIMICROBIAL PEPTIDE, COMPOSITIONS CONTAINING SAME AND USES THEREOF

Background of the Invention

Granulocytes play an important role in host resistance to bacterial, fungal and parasitic pathogens. This resistance is effected through at least two different pathways. In the oxygen dependent pathway, the granulocytes produce toxic oxygen metabolites which participate in killing the invading pathogen. The second pathway is oxygen independent and consists of production and storage of proteins which have potent antimicrobial activity. The importance of these granulocytic defense mechanisms is indicated by the finding that neutropenic patients are unusually susceptible to long term infections, which are sometimes fatal. A number of microbicidal proteins and peptides have been demonstrated in granulocytes, including eosinophil major basic protein, eosinophil cationic protein, bacteria permeability increasing factor and a group of small antibiotic peptides termed defensins which have been isolated from humans (M.E. Selsted et al., J. Clin. Invest. 76: 1436 (1985)), rabbits (M.E. Selsted et al., J. Biol. Chem. 260: 4579 (1985)), and guinea pigs (M.E. Selsted et al., Infec. Immun. 55: 2281 (1987)).

The reported sequences of the known human, rabbit and guinea pig defensins are shown in Figure 4. In addition, U.S. Patent No. 4,543,252, issued September 24, 1985 and assigned to the Regents of the University of California discloses cationic oligopeptides of up to

about 35 amino acids which have microbicidal activity. A comparison of various defensin sequences show that six cysteines are completely conserved among human, rabbit, and guinea pig defensins. It is believed that these residues may be involved in maintenance of secondary structure. In addition, five other residues (2 arg, 2 gly, glu) are conserved in all human and rabbit defensins, three of which are also conserved in guinea pig defensin.

A single difference among human defensins at the N-terminus is sufficient to produce significant changes in microbicidal potency and selectivity (R.I. Lehrer et al., Infect. Immun. 42: 10 (1983)). Moreover, the larger differences among the rabbit defensins drastically alter their bactericidal activity (M.E. Selsted et al., Infect. Immun. 45: 150 (1984)), their candidacidal activity (M.E. Selsted et al., Infect. Immun. 49: 202 (1985)) and their tumor cell cytolytic activity (A. Lichtenstein, Blood 68: 1407 (1986)). Therefore, although sequence conservation suggests certain secondary structures may be necessary for activity, it is clear that these structures are not sufficient for activity and that the potency and the specificity of the peptides are almost solely determined by sequence in non-conserved regions.

The polypeptides of the subject invention shares the cysteine backbone of defensins, but diverges radically in its remaining sequence. It is different in 16 of 34 positions from any of the human or rabbit defensins, and therefore would be expected to differ widely in activity. Moreover, 22 of 33 positions differ from described human defensins. Human defensins are likely to be more important therapeutically because non-human

defensins would be expected to trigger undesirable immune responses.

When tested in vitro the purified polypeptide of this invention has been found to be significantly more active in microbicidal assays against Escherichia coli, Streptococcus faecalis and Candida albicans than a mixture of previously described defensins.

Finally, U.S. Serial No. 125,684, filed November 25, 1987, in the names of Joelle E. Gabay and Carl F. Nathan and assigned to Cornell Research Foundation and the Rockefeller University discloses a series of peaks obtained by reverse-phase, high pressure, liquid chromatography of an extract of human blood. Specifically, Peak 2 of Figure 13 of U.S. Serial No. 125,684 discloses the existence of the polypeptide of this invention. However, it is important to understand that the data presented in U.S. Serial No. 125,684 is that of applicants on the subject application and that this application is assigned to the exclusive licensee of U.S. Serial No. 125,684. Moreover, it is important to note that U.S. Serial No. 125,684 does not disclose that Peak 2 of Figure 13 which contains the polypeptide of this invention has antibacterial or antifungal activity and does not disclose the amino acid sequence or any other characterization of the polypeptide of Peak 2 of Figure 13.

Summary of the Invention

The present invention provides a purified polypeptide useful as an antimicrobial agent having a molecular weight of about 3,700 daltons and the N-terminal amino acid sequence shown in Figure 1 or a biologically active portion thereof. The polypeptide has antimicrobial activity against gram negative and gram positive bacteria and against fungi.

The present invention also provides a method of preparing the purified polypeptide which comprises purification of the polypeptide from granules which in turn are obtained from substantially pure, human granulocytes.

The present invention also provides a pharmaceutical composition comprising an effective bacterial-killing or fungal-killing amount of the purified polypeptide and a pharmaceutically acceptable carrier and the use of this composition to treat subjects having bacterial or fungal infections.

The invention further provides a pharmaceutical composition comprising the purified polypeptide incorporated into a pharmaceutically acceptable liposome and the use of such a composition to treat a subject having a bacterial or fungal infection.

Brief Description of the Figures

Figure 1 - High Performance Reverse-Phase Liquid Chromatography of Granulocyte Extracts

Low molecular weight filtrates from acid extracts of isolated granules were subjected to reverse-phase chromatography as described in Methods. The positions of Peak 1 and Peak 2 is indicated.

Figure 2 - Sequencing Results for Granulocyte Peak 1

Amino acids identified at residues 1-28 by automated sequence analysis of granulocyte Peak 1 (Figure 1).

Figure 3 - Sequencing Results for Granulocyte Peak 2

Amino acids identified at residues 1-33 by automated sequence analysis of granulocyte Peak 2 (Figure 1).

Figure 4 - Sequence Comparison of Granulocyte Peak 2 and Human and Rabbit Defensins

Comparison of the N-terminal sequence determined for granulocyte Peak 2 with published sequences for human, rabbit, and guinea pig defensins.

Figure 5 - Comparison of Amino Acid Compositions of Granulocyte Peak 2 and Human Defensin

Amino acid composition of granulocyte Peak 2 and a mixture of human defensins expressed as mole %. Similarity index = Σ (difference mole %)^2 for each amino acid. Similarity index more than 100 usually indicates unrelated proteins (J.J. Marchalonis et al.,

Comp. Biochem. Physiol. 38: 609 (1971)).

Figure 6 - Comparison of Microbicidal Activity of Granulocyte Peak 1 and Granulocyte Peak 2

Granulocyte Peak 1 and granulocyte Peak 2 were assayed for antimicrobial activity *in vitro* as described in Methods. Amount of peptide required for 50% or 90% killing is compared.

Figure 7 -

Effect of detergents on microbicidal activity of granulocyte Peak 1 and granulocyte Peak 2 is shown.

Detailed Description of the Invention

The present invention provides a purified polypeptide useful as an antimicrobial agent which comprises a polypeptide having a molecular weight of about 3,700 and the N-terminal amino acid sequence val-cys-ser-cys-arg-leu-val-phe-cys-arg-arg-thr-glu-leu-arg-val-gly-asn-cys-leu-ilu-gly-gly-val-ser-phe-thr-tyr-cys-cys-thr-arg-val. The invention further provides biologically active fragments derived from this polypeptide, which as will be readily appreciated by those skilled in the art, may be determined by preparing fragments of the polypeptide using conventional methods.

The invention also concerns DNA molecules which encode the polypeptide of this invention and biologically active fragments thereof and expression vectors which comprise such DNA molecules. Such DNA molecules may be readily prepared using an automated DNA sequence and the well-known codon amino acid relationship of the Genetic Code. Alternatively, such a DNA molecule may be obtained as genomic DNA or as cDNA using oligonucleotide probes and conventional methodologies.

Such DNA molecules may be incorporated into conventional expression vectors or specifically created expression vectors, including plasmids, which are adapted for expression of the DNA and production of the polypeptide in a suitable host such as bacterium, e.g., Escherichia coli, yeast cell, or mammalian cell.

The invention further provides a host vector system for producing a polypeptide having a molecular weight of about 3,700 and the N-terminal amino acid sequence

described above, or a biologically active fragment thereof, which comprises a plasmid containing the DNA sequence encoding the polypeptide in a suitable host.

A method for producing a polypeptide having a molecular weight of about 3,700 and the N-terminal amino acid sequence val-cys-ser-cys-arg-leu-val-phe-cys-arg-arg-thr-glu-leu-arg-val-gly-asn-cys-leu-ilu-gly-gly-val-ser-phe-thr-tyr-cys-cys-thr-arg-val or a biologically active fragment thereof is also provided. The method comprises growing a host vector system comprising an expression vector which contains DNA encoding the polypeptide under suitable conditions permitting production of the polypeptide and recovering the resulting polypeptide from the host.

An alternative method of preparing the purified polypeptide of the invention is also provided. The method comprises:

a) treating human blood cells so as to separately obtain therefrom granulocytes;

b) treating the resulting granulocytes so as to recover therefrom granules;

c) treating the granules so recovered with an extracting agent at a pH less than about 4 so as to separately obtain soluble proteins from the granules;

d) recovering the soluble proteins so separated; and

e) treating the soluble proteins so recovered so as to obtain the purified polypeptide.

In certain embodiments of the invention, the treatment of the soluble proteins comprises size exclusion chromatography, ion-exchange chromatography, or reverse-phase, high performance, liquid chromatography. It will be appreciated by one skilled in the art, however, that treatment of soluble proteins to purify polypeptides may be accomplished by many methods known to those skilled in the art, all of which are contemplated by this invention. Further, in one mebodiment of the invention, the treatment of granulocytes so as to recover granules comprises density gradient centrifugation.

The invention also provides a composition which comprises the purified polypeptide in an amount effective to kill bacteria or fungi and a suitable carrier. Such composition may be used in numerous ways to combat bacteria or fungi, for example, in household or laboratory antimicrobial formulations using carriers well-known in the art.

The invention further provides a pharmaceutical composition for treating a human bacterial or fungal infection which comprises the purified polypeptide of the invention in an amount effective to treat a human bacterial or fungal infection and a pharmaceutically acceptable carrier.

It should be understood that the compositions of the present invention have activity against a wide variety of microorganisms, such as fungi, bacteria (both gram positive and negative), and protozoa and viruses.

Different compositions will have differing degrees of activities towards different organisms. The peptides of the present invention may also be combined with other proteins to act as preservatives to protect the proteins against bacterial degradation. Alternatively, the subject polypeptides or compositions may be used as preservatives and disinfectants in a wide variety of formulations, such as contact lens solutions, ointments, shampoos, medicaments, foods, and the like. The amount of the polypeptide which is employed in the compositions may vary depending upon the nature of the other components, the degree of protection required and the intended use of the composition.

Where the polypeptides are to be used as antimicrobial agents, they can be formulated in buffered aqueous media containing a variety of salts and buffers. The salts will for the most part be alkali and alkaline earth halides, phosphates and sulfates, e.g., sodium chloride, potassium chloride or sodium sulfate. Various buffers may be used, such as citrate, phosphate, HEPES, Tris or the like to the extent that such buffers are physiologically acceptable to the host which is being treated.

Various excipients or other additives may be used, where the compounds are formulated as lyophilized powders, for subsequent use in solution. The excipients may include various polyols, inert powders or other extenders.

Depending on the nature of the formulation and the host, the subject compounds may be administered in a variety of ways. The formulations may be applied topically, by injection, e.g., intravenously, intra-

peritoneally, etc., nasopharyngeally, etc.

The invention further provides a method for killing bacteria or fungi which comprises contacting the bacteria or fungi with an effective amount of the compositions described above. Effective amounts may be readily determined by those skilled in the art.

The invention further provides a method for treating a subject having a bacterial or fungal infection which comprises administering to the subject an effective amount of the pharmaceutical composition described above.

In another aspect of the invention, the composition comprising the purified polypeptide of the invention in an amount effective to kill bacteria or fungi and a suitable carrier; and the pharmaceutical composition for treating a human bacterial or fungal infection which comprises the purified polypeptide of the invention in an amount effective to treat a human bacterial or fungal infection and a pharmaceutically acceptable carrier may additionally comprise a detergent. The addition of a detergent to such compositions is useful to enhance the antibacterial or antifungal characteristics of the novel polypeptide of the invention. Although any suitable detergent may be used, the presently preferred detergent is a nonionic detergent, such as Tween 20 or 1% NP40.

The invention also provides a pharmaceutical composition for treating a human bacterial or fungal infection which comprises the purified polypeptide of the invention in an amount effective to treat a human bacterial or fungal infection incorporated into a

pharmaceutically acceptable liposome.

It will be readily understood by those skilled in the art that any suitable pharmaceutically acceptable liposome may be used as a vehicle for the polypeptide of the present invention. Such liposomal compositions have activity against a wide variety of microorganisms similar to the activity of other compositions of this invention discussed in more detail above. Additionally, these compositions may be administered in a variety of conventional and well-known ways as is also discussed in greater detail above.

The Experimental Detail section which follows is set forth to aid in an understanding of the invention but is not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

Experimental Details

Methods

Isolation of Granulocytes

Buffy coats were obtained from local blood banks, diluted in Hanks Balanced Salts Solution (HBSS), and granulocytes were separated from mononuclear cells by centrifuging 30 ml of diluted cells through 20 ml of 64% Percoll in HBSS (2000 rpm, 20 min.) After aspirating mononuclear cells along with the supernatant, the pellet containing granulocytes was freed of contaminating erythrocytes by hypotonic lysis. Granulocytes were collected by centrifugation (1000 rpm, 10 min) and resuspended in PBS pH 7.0.

Subcellular Fractionation of Granulocytes

Isolated granulocytes in phosphate-buffered saline (2×10^7 cells/ml) were treated with 5 mM diisopropylfluorophosphate (DFP) for 15 minutes at 4°C. The DFP-treated cells were centrifuged at 130 x g for 10 minutes at 4°C, and the resulting pellet was resuspended in an ice-cold buffer containing 100 mM KCl, 3 mM NaCl, 1 mM ATP $(Na)_2$, 3.5 mM $MgCl_2$, and 10 mM Pipes, pH 7.3 (relaxation buffer). The cell suspension was disrupted by nitrogen cavitation for 20 minutes at 350 psi in a bomb (Parr Instrument Company, Moline, Illinois) at 4°C and the cavitate was collected into the Ca^{2+} ion chelater EGTA, pH 7.4, at a final concentration of 1.5 mM. Nuclei and unbroken cells were pelleted (p1) by centrifugation at 500 x g for 10 minutes at 4°C. The postnuclear supernatant (S1) was centrifuged for 15 minutes at 20,000 rpm (SS 34 rotor) on a discontinuous

Percoll density gradient, as described for the isolation of azurophil granules (N.J. Borregaard, J. Cell Biol. 97: 52 (1983)). Fractions of approximately 1 ml were collected at 4°C. Percoll was removed from pooled azurophol granule fractions by centrifugation at 35,000 rpm (180,000 x g) for 2 hours in an SW41 rotor. The layer that sedimented above the packed Percoll was resuspended in relaxation buffer and stored in aliquots at -70°C.

Preparation of Granule Extracts

Fractions from the Percoll gradients corresponding to azurophil granules were pooled and Percoll was removed by centrifugation as described above. The granule preparation was resuspended in relaxation buffer and stored at -70°C. The isolated granules were extracted with vigorous agitation in 0.05 M glycine-HCl buffer pH 2.0 for 40 minutes at 25°C. The acid-extract was centrifuged at 30,000 x g for 20 minutes to obtain a soluble fraction. The soluble fraction thus obtained was concentrated by centrifugation in Centricon 10 units (Amicon). The filtrate was used as starting material for chromatographic purification.

Microbicidal Assays

Microbicidal activity was routinely tested against *Escherichia coli* K12 (MC4100), *Streptococcus faecalis* (ATCC Accession No. 29212) and *Candida albicans* (clinical isolate). Organisms from a single colony on agar plates were inoculated into liquid medium and cultured overnight at 37°C. Aliquots of the overnight culture were inoculated into fresh nutrient broth and grown to mid-exponential phase. Cultures were then

diluted into the test medium to the appropriate concentration.

Samples from reverse-phase liquid chromatography to be tested for microbicidal activity were dried by vacuum centrifugation in Immulon I microtire plates in the presence of 50 μl 0.1% BSA. Dried samples were resuspended in 0.1% acetic acid and returned to dryness by vacuum centrifugation. Samples were then resuspended in assay buffer and after appropriate dilutions of samples had been made, test organisms were added to the wells and incubation at 37°C was carried out for 30 to 60 minutes. Killing of all organisms was carried out in 20 mM phosphate pH 6, 0.25 M glucose and 0.02% Tween 20 unless otherwise indicated. A volume of the assay mixture corresponding to 200-300 organisms in the control assays were plated onto trypticase soy agar plates (<u>Escherichia coli</u> and <u>Streptococcus faecalis</u>) or spread onto Sabouraud dextrose agar plates (<u>Candida albicans</u>). Colony forming units were determined manually or by automated counting after overnight incubation at 37°C.

<u>Characterization of Centricon Filtrates by Reverse-Phase, High Performance, Liquid Chromatography</u>

Trifluoracetic acid (TFA) was added to Centricon 10 Filtrates to 0.1% and the samples were applied to a Vydac wide pore C4 (250 x 4 mm) reverse-phase column and run on the gradient described below. Solvent A was 0.1% aqueous TFA and Solvent B was 0.1% TFA in HPLC grade acetonitrile. The gradient was as follows:

TIME	% SOLVENT B
0-2 minutes	0%
2-7 minutes	0-15%
7-67 minutes	15-50%
67-72 minutes	50-100%
72-75 minutes	100%

The equipment utilized was a Beckman HPLC system consisting of a Vydac wide pore C4 (250 x 4 mm) reverse-phase column, two 110B pumps, a 421A controller, a 210A injector, a 2 ml sample loop, a 163 variable wavelength detector, a 2112 Redirac fraction collector, and a Kipp and Zonen BD 41 chart recorder. The detector setting was 214 nm, 0-2.0 absorbance units full scale (AUFS) and the peak fractions were collected manually.

Sequence Analysis of Peak 1 and Peak 2 Peptides

Reversed phase HPLC purified Peak 1 and Peak 2 were concentrated to 50 microliters on a Speed Vac and loaded onto an Applied Biosystems 477A pulse liquid phase sequenator. Phenylthiohydantoin (PTH) analysis was performed on line using an Applied Biosystems Model 120A PTH Analyzer.

Amino Acid Analysis of Peak 1 and Peak 2

PTC amino acid analysis of polypeptides was obtained by 1 hour hydrolysis with 6.0 N HCl at 150°C using a Waters Picotag system equipped with a Bckman HPLC system.

Results

Granulocytes were isolated from buffy coats and protein was extracted from a Percoll-purified granule fraction as described in Methods. When this protein extract was concentrated by ultrafiltration in Centricon 10 units (Amicon), a filtrate was obtained which contained a portion of the low molecular weight proteins/peptides in the extract.

This filtrate was analyzed by high performance reverse-phase liquid chromatography on a Vydac C4 column as described in Methods. Under these conditions, two major peaks were detected by absorbance at 214 nm (Figure 1).

When amino acid sequence analysis was performed as described in Methods, the early eluting peak (Peak 1) was determined to be a mixture of three known human defensins (Figure 2). Estimating from N-terminal yields on sequencing, about 7% of the mixture was HNP3 and the remainder was almost evenly distributed between HNP1 (44%) and HNP2 (49%). Sequence analysis showed that the later eluting peak (Peak 2) contained the single sequence idnicated in Figure 3. Computer homology search of the Swiss Pro database (Intelligenetics) using the Needleman-Wunsch method identified the similarity Peak 2 and the cysteine backbone of the human defensins (S.B. Needleman and D.D. Wunsch, J. Mol. Biol. 48: 443 (1970)). The sequence outside this cysteine backbone radically diverged from reported defensin sequences. Computer search failed to identify significant homology with any other known protein. Comparison of the amino acid compositions for Peak 1 and Peak 2 (Figure 5) also

demonstrates major differences bgetween Peak 2 and Peak 1.

Aliquots of the reverse-phase peaks were tested for microbicidal activity in vitro against Escherichia coli, Streptococcus faecalis and Candida albicans as described in Methods. Protein concentrations in the samples were determined by amino acid composition analysis. The results (Figure 6) clearly demonstrate that the Peak 2 peptide has much greater microbicidal activity than defensins (Peak 1). The higher specific activity of Peak 2 is particularly evident for activity against the gram positive organism Streptococcus faecalis, where the concentration required to reach the indicated levels of killing is about 100 fold less than for Peak 1. Peak 2 peptide is also more active against the gram negative bacteria Escherichia coli (15-30 fold) and the fungus Candida albicans (about 5 fold). It is clear that the altered sequence between Peak 2 and the defensins causes major alterations in bactericidal and fungicidal potencies.

Additional studies were performed on the effects of the detergents Tween 20 and NP40 on the activities of Peak 1 and Peak 2 (Figure 7). These detergents enhanced the activities of both peaks against bacteria and to a lesser extent against Candida albicans.

What is claimed is:

1. A purified polypeptide useful as an antimicrobial agent having a molecular weight of about 3,700 and the N-terminal amino acid sequence val-cys-ser-cys-arg-leu-val-phe-cys-arg-arg-thr-glu-leu-arg-val-gly-asn-cys-leu-ilu-gly-gly-val-ser-phe-thr-tyr-cys-cys-thr-arg-val, or a biologically active fragment derived therefrom.

2. A DNA molecule which encodes the polypeptide of claim 1.

3. An expression vector which comprises the DNA molecule of claim 2.

4. A plasmid of claim 3.

5. A host vector system for producing a polypeptide having a molecular weight of about 3,700 and the N-terminal amino acid sequence val-cys-ser-cys-arg-leu-val-phe-cys-arg-arg-thr-glu-leu-arg-val-gly-asn-cys-leu-ilu-gly-gly-val-ser-phe-thr-tyr-cys-cys-thr-arg-val, or a biologically-active fragment thereof, which comprises the plasmid of claim 4 in a suitable host.

6. A method for producing a polypeptide having a molecular weight of about 3,700 and the N-terminal amino acid sequence val-cys-ser-cys-arg-leu-val-phe-cys-arg-arg-thr-glu-leu-arg-val-gly-asn-cys-leu-ilu-gly-gly-val-ser-phe-thr-tyr-cys-cys-thr-arg-val, or a fragment

thereof, which comprises growing the host vector system of claim 5 under suitable conditions permitting production of the polypeptide, and recovering the resulting polypeptide.

7. A method for preparing a purified polypeptide of about 3,700 daltons having the N-terminal amino acid sequence val-cys-ser-cys-arg-leu-val-phe-cys-arg-arg-thr-glu-leu-arg-val-gly-asn-cys-leu-ilu-gly-gly-val-ser-phe-thr-tyr-cys-cys-thr-arg-val which comprises:

a) treating human blood cells so as to separately obtain therefrom granulocytes;

b) treating the resulting granulocytes so as to recover therefrom granules;

c) treating the granules so recovered with an extracting agent at a pH less than about 4 so as to separate soluble proteins from the granules;

d) recovering the soluble proteins so separated; and

e) treating the soluble proteins so recovered so as to obtain the purified polypeptide.

8. A method according to claim 7, wherein the treatment of the soluble proteins comprises size exclusion chromatography.

9. A method according to claim 7, wherein the treatment of the soluble proteins comprises ion-exchange chromatography.

10. A method according to claim 7, wherein the treatment of the granulocyte so as to recover granules comprises density gradient centrifugation.

11. A method according to claim 7, wherein the treatment of the soluble proteins comprises reverse-phase, high performance, liquid chromatography.

12. A composition which comprises the purified polypeptide of claim 1 in an amount effective to kill bacteria or fungi and a suitable carrier.

13. A pharmaceutical composition for treating a human bacterial or fungal infection which comprises the purified polypeptide of claim 1 in an amount effective to treat the human bacterial or fungal infection and a pharmaceutically acceptable carrier.

14. A method for killing bacteria or fungi which comprises contacting the bacteria or fungi with an effective amount of the composition of claim 12.

15. A method for treating a subject having a bacterial or fungal infection which comprises administering to the subject an effective amount of

the pharmaceutical composition of claim 13.

16. A composition of claim 12 or 13 which further comprises a detergent.

17. A composition of claim 16, wherein the detergent is nonionic.

18. A pharmaceutical composition for treating a human bacterial or fungal infection which comprises the purified polypeptide of claim 1 in an amount effective to treat the human bacterial or fungal infection and a pharmaceutically acceptable liposome.

FIGURE 1

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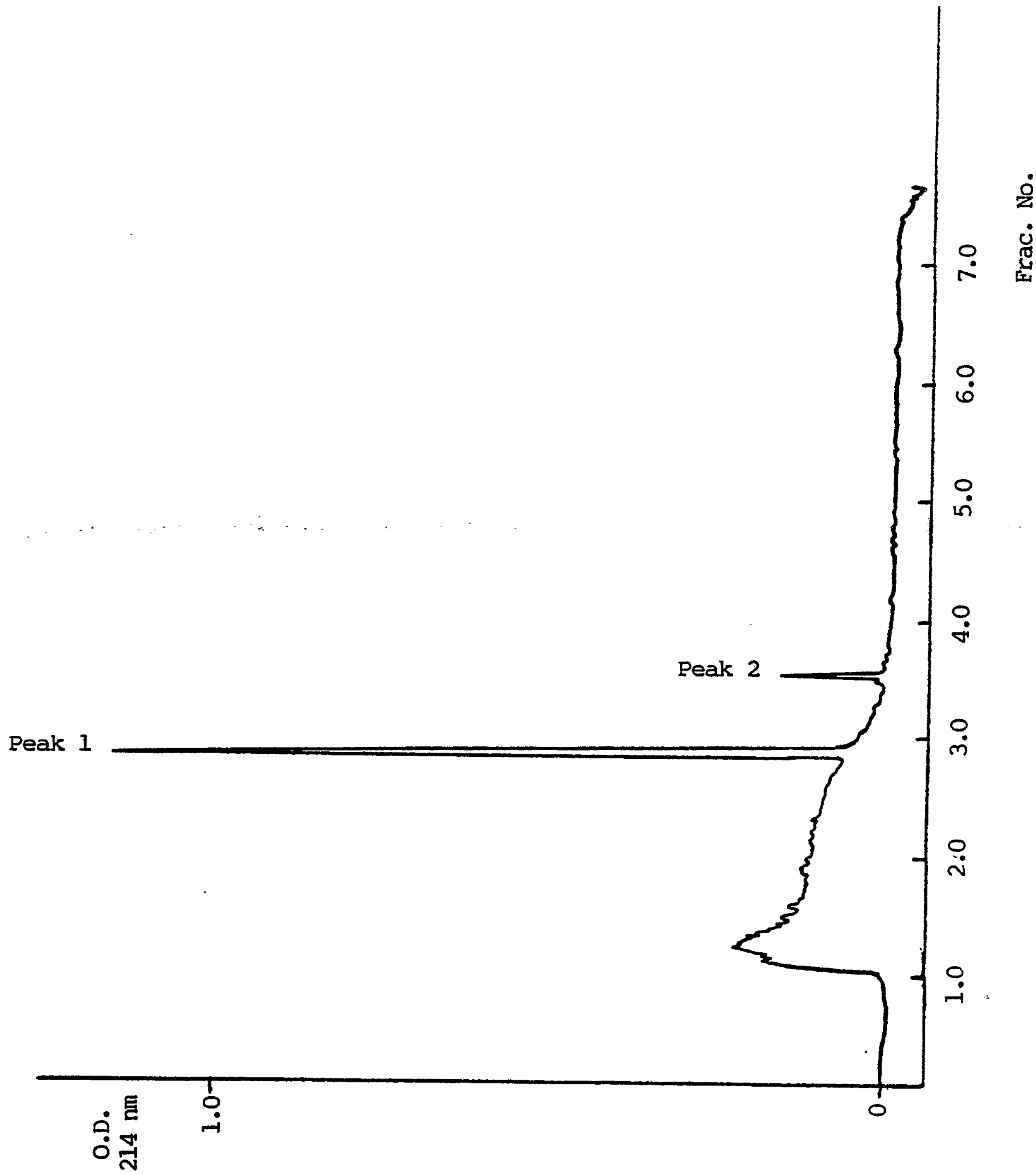

FIGURE 2
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N-TERMINAL
AMINO ACID SEQUENCE OF PEAK 1

RESIDUE	HNP1 AND 3*	YIELD IN PICOMOLES	HNP2*	YIELD IN PICOMOLES
1	ALA/ASP	518/86	---	---
2	---	---	TYR	581
3	TYR	640	---	---
4	---	---	ARG	151
5	ARG	65	ILE	460
6	ILE	757	PRO	348
7	PRO	427	ALA	363
8	ALA	310	---	---
9	---	---	ILE	353
10	ILE	493	ALA	224
11	ALA	607	GLY	267
12	GLY	450	GLU	204
13	GLU	105	ARG	31
14	ARG	446	---	---
15	ARG	424	TYR	258
16	TYR	479	GLY	229
17	GLY	295	THR	105
18	THR	232	---	---
19	---	---	ILE	177
20	ILE	301	TYR	175
21	TYR	271	GLN	122
22	GLN	225	GLY	115
23	GLY	144	ARG	52
24	ARG	101	LEU	80
25	LEU	83	TRP	15
26	TRP	34	ALA	24
27	ALA	109	PHE	46
28	PHE	90	---	---

* As defined in Selsted, M.E. et al. (1985) J. Clin. Invest. 76, 1436.

FIGURE 3
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N-TERMINAL AMINO ACID
SEQUENCE OF PEAK 2

RESIDUE	PEAK 2	YIELD IN PICOMOLES
1	VAL	212
2	---	---
3	SER	85
4	---	---
5	ARG	46
6	LEU	116
7	VAL	133
8	PHE	135
9	---	---
10	ARG	72
11	ARG	92
12	THR	88
13	GLU	62
14	LEU	82
15	ARG	47
16	VAL	42
17	GLY	69
18	ASN	68
19	---	---
20	LEU	48
21	ILE	43
22	GLY	46
23	GLY	59
24	VAL	30
25	SER	25
26	PHE	26
27	THR	21
28	TYR	25
29	---	---
30	---	---
31	THR	9
32	ARG	8
33	VAL	4

FIGURE 4
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COMPARISON OF PEAK 2 TO HUMAN,
RABBIT, AND GUINEA PIG DEFENSINS

		1				5					10			
PEAK 2		VAL	CYS	SER	CYS	ARG	LEU	VAL	PHE	CYS	ARG	ARG	THR	GLU
HNP-1**		ALA	CYS	TYR	CYS	ARG	ILE	PRO	ALA	CYS	ILE	ALA	GLY	GLU
HNP-2			CYS	TYR	CYS	ARG	ILE	PRO	ALA	CYS	ILE	ALA	GLY	GLU
HNP-3		ASP	CYS	TYR	CYS	ARG	ILE	PRO	ALA	CYS	ILE	ALA	GLY	GLU
RNP-1***	VAL	VAL	CYS	ALA	CYS	ARG	ARG	ALA	LEU	CYS	LEU	PRO	ARG	GLU
RNP-2	VAL	VAL	CYS	ALA	CYS	ARG	ARG	ALA	LEU	CYS	LEU	PRO	LEU	GLU
RNP-3a	GLY	ILE	CYS	ALA	CYS	ARG	ARG	ARG	PHE	CYS	PRO	ASN	SER	GLU
RNP-3b	GLY	ARG	CYS	VAL	CYS	ARG	LYS	GLN	2LEU*	CYS	SER	TYR	ARG	GLU
RNP-4	VAL	SER	CYS	THR	CYS	ARG	ARG	PHE	SER	CYS	GLY	PHE	GLY	GLU
RNP-5	VAL	PHE	CYS	THR	CYS	ARG	GLY	PHE	LEU	CYS	GLY	SER	GLY	GLU
GPNP****	ARG	ARG	CYS	ILE	CYS	THR	THR	ARG	THR	CYS	ARG	PHE	PRO	TYR

		15					20					25		
PEAK 2	LEU	ARG	VAL	GLY	ASN	CYS	LEU	ILE	GLY	GLY	VAL	SER	PHE	THR
HNP-1	ARG	ARG	TYR	GLY	THR	CYS	ILE	TYR	GLN	GLY	ARG	LEU	TRP	ALA
HNP-2	ARG	ARG	TYR	GLY	THR	CYS	ILE	TYR	GLN	GLY	ARG	LEU	TRP	ALA
HNP-3	ARG	ARG	TYR	GLY	THR	CYS	ILE	TYR	GLN	GLY	ARG	LEU	TRP	ALA
RNP-1	ARG	ARG	ALA	GLY	PHE	CYS	ARG	ILE	ARG	GLY	ARG	ILE	HIS	PRO
RNP-2	ARG	ARG	ALA	GLY	PHE	CYS	ARG	ILE	ARG	GLY	ARG	ILE	HIS	PRO
RNP-3a	ARG	PHE	SER	GLY	TYR	CYS	ARG	VAL	ASN	GLY	ALA	ARG	TYR	VAL
RNP-3b	ARG	ARG	ILE	GLY	ASP	CYS	LYS	ILE	ARG	GLY	VAL	ARG	PHE	PRO
RNP-4	ARG	ALA	SER	GLY	SER	CYS	THR	VAL	ASN	GLY	VAL	ARG	HIS	THR
RNP-5	ARG	ALA	SER	GLY	SER	CYS	THR	ILE	ASN	GLY	VAL	ARG	HIS	THR
GPNP	ARG	ARG	LEU	GLY	THR	CYS	ILE	PHE	GLN	ASN	ARG	VAL	TYR	THR

			30			
PEAK 2	TYR	CYS	CYS	THR	ARG	VAL
HNP-1	PHE	CYS	CYS			
HNP-2	PHE	CYS	CYS			
HNP-3	PHE	CYS	CYS			
RNP-1	LEU	CYS	CYS	ARG	ARG	
RNP-2	LEU	CYS	CYS	ARG	ARG	
RNP-3a	ARG	CYS	CYS	SER	ARG	ARG
RNP-3b	PHE	CYS	CYS	PRO	ARG	
RNP-4	LEU	CYS	CYS	ARG	ARG	
RNP-5	LEU	CYS	CYS	ARG		
GPNP	PHE	CYS	CYS			

* leu - leu bond expressed as 2leu
** HNP - human neutrophil peptide
*** RNP - rabbit neutrophil peptide
**** GPNP - guinea pig neutrophil peptide

FIGURE 5
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AMINO ACID COMPOSITION
COMPARISON BETWEEN PEAK 1 AND PEAK 2

	PEAK 1	PEAK 2	DIFFERENCES SQUARED
ASP	0.97	2.39	2.02
GLU	5.41	2.44	8.82
SER	0.00	4.80	23.04
GLY	10.02	7.66	5.57
HIS	0.00	0.00	0.00
ARG	17.24	14.74	6.25
THR	2.98	8.26	27.88
ALA	9.64	0.25	88.17
PRO	4.42	0.00	19.54
TYR	6.04	2.78	10.63
VAL	0.33	10.82	110.04
MET	0.00	0.00	0.00
ILE	7.32	2.23	25.91
LEU	2.95	6.71	14.14
PHE	3.02	4.56	2.37
LYS	0.00	0.00	0.00

SIMILARITY INDEX = 344.87

FIGURE 6
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COMPARISON OF MICROBICIDAL ACTIVITY OF GRANULOCYTE PEAK 1 AND GRANULOCYTE PEAK 2

		E. COLI	*S. FAECALIS*	*C. ALBICANS*
PEAK 1 (μg)	90% KILL	>3.6	>3.6	>3.6
	50% KILL	0.7	>3.6	1.2
PEAK 2 (μg)	90% KILL	0.25	0.04	0.9
	50% KILL	0.02	0.02	0.2

FIGURE 7
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EFFECT OF DETERGENTS ON MICROBICIDAL ACTIVITY OF GRANULOCYTE PEAK 1 AND GRANULOCYTE PEAK 2

		GREATEST DILUTION TO GIVE 90% KILL		
		NO DETERGENT	.02% TWEEN 20	1% NP40
PEAK 1				
	E. COLI	40	80	80
	S. FAECALIS	<20	<20	80
	C. ALBICANS	20	80	20
PEAK 2				
	E. COLI	40	>160	>160
	S. FAECALIS	40	80	>160
	C. ALBICANS	<20	20	<20

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/02317

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC
IPC(4):A61K 37/02; C07K 1/14, 3/02, 3/20, 15/06; C12P 21/00
U.S. CL.:514/12; 530/324,344; 536/27; 435/68,320,240.1

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S.	514/12; 530/324,344; 536/27; 435/68, 320, 240.1

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

Databases: Biosis, Chemical Abstract Service (CAS), Automated Patent Search (APS, USPat), Sequence search (Genbank,EMBL)

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
A	US, A, 4,705,777 (Lehrer) 10 January 1987. See entire article.	1-18
T	N, Journal of Biological Chemistry, 05 July 1989. V 264(19) pp. 11200-11203, Wilde. See entire article.	1-18

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22 August 1989	13 SEP 1989
International Searching Authority	**Signature of Authorized Officer**
ISA/US	NINA OSSANNA, Ph.D.

Form PCT/ISA/210 (second sheet) (Rev.11-87)

International Application No. PCT/US89/02317

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [ ] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [ ] Claim numbers .... , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. [ ] Claim numbers .... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. [ ] Claim numbers____________, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [X] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See attachment.

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. [X] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did ~~not~~ invite payment of any additional fee, but reconsidered.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

PCT/US89/02317

Attachment to Form PCT/ISA/210

LACK OF UNITY

I. Claims 1, 7-14, 16 and 17, drawn to a polypeptide, method of making polypeptide and method of using polypeptide; class 530/324, class 530/344 and class 514/12.

II. Claims 2-6, drawn to DNA, molecule, plasmid and expression vector, host-vector system and method of making polypeptide; class 536/27, class 435/240.2 and 435/68.

III. Claim 15, drawn to method of treating humans; class 514/12.

IV. Claim 18, drawn to composition with liposomes; class 514/12.